GENE THERAPY OF CANCER USING AN INTERLEUKIN 2 TRANSMEMBRANE CONSTRUCT

by

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FINAL READING APPROVAL

To the Graduate Council of the University of Utah:

I have read the thesis of Nathan Bradley Adams in its final form and have found that (1) its format, citations, and bibliographic style are consistent and acceptable; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the supervisory committee and is ready for submission to The Graduate School.

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ABSTRACT

Selective expression of cytokines on the surface of tumors is likely to stimulate tumor-infiltrating lymphocytes that are primed and already recognize tumor antigens. This may result in enhanced tumor recognition and killing. This gene therapy approach may avoid toxicity associated with systemic therapy with high doses of the cytokines needed to achieve the same effects. In order to test this concept, we have evaluated an IL-2 gene construct that is designed to induce tumor cells to express a membrane bound form of IL-2 (IL-2tm). A mammalian plasmid expression vector was designed to express a fusion gene consisting of human interleukin 2 with a Fcε-γ transmembrane anchor derived from a subunit of the FC epsilon receptor. We demonstrated that mRNA and protein for the IL-2tm fusion protein was expressed in transfected RD995 tumor cells. Expression of the IL-2tm protein on the tumor cell surface membrane was confirmed by laser confocal microscopy. In order to assess biological function, RD995 transfected with IL-2tm or pCMV2b (empty expression vector) were implanted subcutaneously into C3H/HEN mice. Nontransfected RD995 was also implanted subcutaneously for tumor growth comparison (control group). RD995 tumor cells (10^6 or 10^5 /mouse) transfected with IL-2tm grew more slowly than RD995 transfected with pCMV2b or non-transfected control RD995 implanted tumors after implantation into syngenic animals. Further preclinical evaluation of the IL-2tm gene therapy as a possible cancer treatment is underway.

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INTRODUCTION

Interleukin 2 is a cytokine produced primarily by T cell lymphocytes that has been shown to have anti-tumor effects.[1] Human IL-2 is a 15-kD glycoprotein composed of 133 amino acids.[2-3] Produced primarily by CD4+ helper T cells, the cytokine consists of four antiparallel alpha helices that are connected by three loops. IL-2 was first described as a T cell growth factor for antigen-activated T cells.[2] IL-2 is necessary for induction and clonal expansion of antigen-specific cytotoxic T cells. In addition, this cytokine is a differentiation-maturation factor for B cells and T cells.[18] At high concentrations (>600 IU/ml), IL-2 is also the principle cytokine responsible for inducing NK cell derived lymphokine-activated killer (LAK) cells. Cytotoxic T and NK lymphocytes are believed to be critical for recognition of aberrant or malignant cells and eradication of tumors.[1, 3-4,12,16]

These antitumor properties provide the basis for IL-2 therapy of cancer.[14] Intravenous administration of recombinant human IL-2 is FDA approved for treatment of advanced melanoma and renal cell carcinoma.[17] IL-2 immunotherapy produces responses in about 15% of melanoma patients and 20% in renal cell carcinoma patients.[5] Interestingly, 5-10% of patients that respond to IL-2 immunotherapy experience a durable complete response with up to 15 years follow-up.[6] However, IL-2 therapy produces severe dose-dependent toxicity that often prevents the patient from completing the IL-2

treatment regimen.[7] Side effects include weight gain, ascites, dyspnea, pulmonary edema and severe hypotension.

Efforts to circumvent these side effects have led to IL-2 based gene therapy protocols.[14] Tumor cells have been engineered to secrete IL-2 into the microenvironment surrounding the tumor. Most tumors contain infiltrating CD8+ lymphocytes.[23-24] When these cells are initially isolated from tumors, they can be activated and clonally expanded in the presence of IL-2 to generate tumor specific cytotoxic lymphocytes. Thus, a goal of gene therapy is to cause intratumoral secretion of activating cytokines resulting in enhanced activation of cytotoxic tumor-infiltrating lymphocytes (TIL) within tumors.[8] Examples of successful IL-2 based gene therapy have been published.[19] Horton et al. evaluated IL-2 gene therapy of murine ovarian cancer.[9] Murine ovarian tumors (MOT) were treated with an IL-2 plasmid DNA complexed with the cationic lipid, N-(2-hydroxyethyl)-N,N-dimethyl-2-3-bis(tetradecyloxy)-1-propanaminium bromide/dioleoylphosphatidylethanolamine (DMRIE/DOPE). MOT tumor-bearing mice injected i.p. with IL-2 plasmid:DMRIE/DOPE on days 5, 8, and 11 after tumor cell implantation demonstrated a significant inhibition of tumor ascites as well as a significant increase in survival. By day 26 after tumor cell injection, 10% of the mice treated with the control pDNA were still alive compared with 70% of the mice treated with IL-2 pDNA:DMRIE/DOPE. Furthermore, the peritoneal fluid of mice treated with IL-2 pDNA: DMRIE/DOPE had an IL-2 specific increase in the levels of interferon gamma (IFN-γ) and granulocyte stimulating factor (GM-CSF). This study also investigated the nature of the immune response to the MOT cells, using nude mice (immunodeficient mice without a thymus and without T cells). Only a modest inhibition of tumor growth

occurred in nude mice, showing that T cells are required for IL-2 gene mediated anti-tumor effects.[9]

We and others have pioneered a new approach to gene therapy by creating a fusion gene encoding a cytokine plus a transmembrane domain. Cell membrane-expressed cytokines may be able to activate immune cells in close proximity to tumor antigens that act to create a specific immune recognition. An added advantage is that low level of cytokine expression remains localized to the tumor and is not associated with the toxicity of high systemic doses of the cytokine.[10-11] There are two published studies of membrane bound cytokines (not IL-2). In the first paper, murine tumor necrosis factor alpha (TNFα) was expressed in a membrane bound (nonsecreted) form by Marr using an adenoviral vector in a murine transgenic breast cancer model. They were able to reduce systemic toxicity with little or no reduction of antitumor activity.[10]

In a second paper, membrane-bound forms of GM-CSF and IFN-γ were created.[11] Cloned GM-CSF and IFN-γ were both bound to a transmembrane domain (gamma single chain of the FcεRI). El-Shami showed that surface co-expression of GM-CSF and IFN-γ induced a cytotoxic T lymphocyte (CTL) response that protected syngenic mice against D122 (clone of Lewis lung carcinoma). Surface co-expression of GM-CSF/IFN-γ also protected mice from subsequent rechallenge of nontransfected D122 tumor cells. The researchers also reported that toxic side effects were not detected in the mice.[11]

The purpose of this thesis is to engineer and evaluate a plasmid containing a transgene encoding membrane bound IL-2 (IL-2tm). By inducing expression of IL-2 on the surface of tumor cells, we hypothesize that IL-2 will activate tumor infiltrating lymphocytes in proximity to tumor antigens. This would increase activation of antigen-

specific T cells and result in destruction of tumor cells expressing tumor-associated antigens. The IL-2tm construct was created by joining the gene for human IL-2 with the transmembrane domain Fcε-γ as previously described by El-Shami.[11]

The three objectives of this thesis project were: 1) construction of a plasmid to express membrane bound IL-2 protein on the surface of mammalian tumor cells, 2) evaluation of the capacity of tumor cells transfected with IL-2tm containing plasmid to induce mRNA and protein expression and 3) assessment of biological anti-tumor activity of RD995 tumor cells transfected with the hIL-2+ Fcε-γ gene construct.

MATERIALS AND METHODS

Insertion of human interleukin 2 into cloning vector pBluescript KS and sequence verification

Plasmid VR1103 containing the human Interleukin 2 (hIL-2) gene was a gift from Vical, Inc. Restriction enzymes PstI and BamHI (Life Technologies, Gaithersburg, MD) were used to excise the hIL-2 gene from the plasmid. The cut VR1103 plasmid was run on a low melting point 1% agarose gel (Sigma Chemical Company, St. Louis, MO) and the band containing the IL-2 gene was identified and isolated from the gel using GeneCAPSULE ™ (Geno Technology, Inc., St. Louis, MO). The expected DNA size was 616 base pairs. The identity of the gene was confirmed by direct DNA sequencing (Huntsman Cancer Institute Core Facility, University of Utah, Salt Lake City, UT).

Cloning vector pBluescript II KS (+/-) (Stratagene, La Jolla, CA) was cut with PstI and BamHI (Life Technologies, Gaithersburg, MD) and run on a low melting point 1% agarose gel (Sigma Chemical Company, St. Louis, MO). pBluescript KS was isolated from the gel to remove the segment of DNA between PstI and BamHI (the stuffer portion). hIL-2 was then ligated into pBluescript KS, by combining 0.5 units of T4 DNA Ligase (Boehringer Mannheim, GmbH, Germany), 5 µl hIL-2 gene (23 ng/µl) insert with 2 µl of cut pBluescript KS (150 ng/ µl). DH5α ™ Competent Cells (Life Technologies, Gaithersburg, MD) were transformed with the ligated cloning vector following the protocol in the package insert. Transformed DH5α ™ *E. coli* was plated on

ampicillin agar plates (100 μg/ml) for isolation. Selection of bacterial colonies expressing hIL-2 was performed using lacZ color selection of recombinant plasmids. Sterile toothpicks were used to select individual white colonies. The colonies were then grown in LB media with ampicillin (100 μg/ml) for 16 h. A Qiagen ™ Plasmid Midi kit (Valencia, CA) was used to isolate hIL-2/pBluescript KS. The insertion of intact hIL-2 gene was verified by DNA based PCR, using the primers (5' TGC TGG ATT TAC AGA TGA TTT 3') and (5' CAC TTC CTC CAG AGG TTT G 3'). PCR was run for 32 cycles at 55° C annealing for 15 sec, 72° C extension for 30 sec resulting in a 155 base pair product, which was verified by direct sequencing.

Removal of stop codon using PCR directed mutagenesis

The stop codon in the IL-2 gene was identified. Primers were designed to remove this stop codon and add restriction sites BamHI (5' GGA TCC 3') on the sense strand and EcoRI (5' GAA TTC 3') on the antisense strand in order to facilitate insertion into a mammalian expression vector. The following primers were used (sense 5' AGA ACT AGT GGA TCC GCA CCT ACT TCA AGT TCT 3') and (antisense 5' GTC AGG GAA TTC AGT CAG TGT TGA GAT GCT TTG 3'). PCR of hIL-2/pBluescript KS was performed using the following parameters: 30 cycles at 60° C annealing for 30 sec, 72° C extension for 120 sec, using Herculase™ Enhanced DNA Polymerase (Stratagene, La Jolla, CA). The resulting 423 bp PCR product was isolated from a 1% agarose gel as described above. Restriction enzymes BamHI and EcoRI were then used to digest the isolated hIL-2 gene. The digested hIL-2 PCR product was separated on a 1% agarose gel and isolated as previously described.

Construction of a hIL-2 fusion gene containing a transmembrane domain from Fcε-γ

Fcε-γ was chosen to donate a transmembrane domain based on a previously published study by El-Shami. [11] HTAAA91 plasmid encoding Fcε- γ was purchased from ATCC (Manassas, VA). The Fcε-γ gene was excised from the plasmid using restriction enzymes BamHI and EcoRI. Ligation of Fcε-γ and modified hIL-2 was performed under the following conditions: 0.5 units of T4 DNA Ligase (Boehringer Mannheim, GmbH, Germany), 5 µl of gel extracted Fcε-γ, 2 µl of hIL-2 gene and 8 µl of DEPC treated water. DH5α ™ Competent Cells were transformed with the ligation product and grown on ampicillin agar selection plates. The colonies were screened by PCR using primers spanning the fusion product of the IL-2 gene and the transmembrane region of Fcε-γ (5' TGC TGG ATT TAC AGA TGA TTT 3') and (5' CAC TTC CTC CAG AGG TTT G 3'). PCR conditions were 32 cycles at 55° C annealing for 15 sec, 72° C extension for 30 sec. The PCR products were run on a 1% agarose gel and colonies containing hIL-2/ Fcε- γ (IL-2tm) fusion product were chosen for expansion and grown overnight in LB ampicillin broth and isolated with the Qiagen ™ Plasmid Midi kit (Valencia, CA). The plasmids were sequenced using PCR based sequencing to verify proper insertion of hIL-2and to ensure removal of the stop codon.

Ligation of hIL-2/Fcε- γ fusion gene into the mammalian expression vector pCMV2b

The pCMV2b (Stratagene, La Jolla, CA) expression vector was selected to maintain in frame transcription of the hIL-2/Fcε- γ gene. This vector contains a cytomegalovirus promoter along with neomycin and kanamycin resistance selection

markers. pCMV2b is an N-terminal FLAG® tagging vector. The FLAG® protein was included to facilitate tracking of the fusion protein, using a FLAG specific antibody.

pCMV2b vector and hIL-2/Fcε- γ were cut with ApaI and BamHI (Life Technologies, Gaithersburg, MD) and gel isolated as previously described. Ligation of the vector and gene was performed under the following conditions: 1 unit of T4 DNA Ligase (Boehringer Mannheim, GmbH, Germany), 2 μl of ligation buffer (provided in kit), 14 μl of ApaI and BamHI cut pCMV2b, 2 μl of ApaI and BamHI cut hIL-2/ Fcε- γ and 3 μl of DEPC treated water. DH5α ™ Competent Cells (Life Technologies, Gaithersburg, MD) were transformed with the resulting plasmid and grown on agar plates containing 50 μg/ml kanamycin. The colonies were screened using the same screening procedure described previously. Lane 2 was isolated from the gel and sequenced using T3 and T7 based primers to verify proper insertion of hIL-2/Fcε- γ into the expression vector pCMV2b. pCMV2b + hIL-2/Fcε- γ is designated the IL-2tm vector.

Transfection of IL-2tm and pCMV2b into murine tumor cells RD995

RD995 cells were cultured in six-well plates at a concentration of 10^5 cells per well and allowed to adhere overnight. In order to determine the optimum Lipofectin and DNA concentrations, Lipofectin (1 mg/ml) (Life Technologies, Gaithersburg, MD) was used to transfect RD995 at the following volumes: 2 μl, 10 μl, and 20 μl along with either 1.0 μg or 2.0 μg of IL-2tm using the procedure described in the Lipofectin package insert. Cells transfected with pCMV2b empty vector served as a control. Optimum Lipofectin/DNA concentration was determined based on the number of RD995 surviving selection with 800 μg/ml G418 (Sigma Chemical Company, ST. Louis, MO).

Screening for IL2tm and pCMV2b mRNA expression

Using predetermined optimal conditions, RD995 cells were transfected with IL-2tm (2 μg) or pCMV2b empty vector (Lipofectin 10 μl) and allowed to grow for 1 week. They were then transferred to 200 ml culture flasks (Corning Costar Corporation, Cambridge, MA) containing 30 ml of RPMI-1640 (BioWhittaker, Walkersville, MD) culture media supplemented with 5% fetal calf serum.

After expanding RD995 cultures for 1 week, 800 μg/ml G418 (Sigma Chemical Company, ST. Louis, MO) was added to cultures to select for tumor cells expressing the IL-2tm plasmid or the pCMV2b plasmid. Untransfected control cells were universally killed by this concentration of G418. Transfected cells were allowed to grow for an additional 2 to 3 weeks. Total RNA was subsequently isolated from 10^6 cultured cells using TRI reagent (Molecular Research Center, INC., Cincinnati, OH). In order to remove residual plasmid DNA, the isolated RNA was treated with 0.6 μl of DNase (Life Technologies, Gaithersburg, MD) followed by phenol/chloroform extraction. Reverse transcription of mRNA was performed under the following conditions: 200 ng total RNA, 1.0 μl M-MLV-reverse transcriptase (200 units/μl), 1 hr incubation at 37 ° C. PCR was performed on the IL-2tm and pCMV2b cDNA using primers spanning the IL-2 gene and the transmembrane fusion domain as previously described.

Western blot analysis of IL-2tm protein expression

in RD995 tumor cells

One million RD995 cells transfected with IL-2tm using (2.0 μg of IL-2tm and 10μl of Lipofectin) were grown in a six well plate containing 5 ml of RPMI-1640 culture

media without G418. Tumor cells were harvested at days 1-5. One million harvested cells were washed twice in PBS and lysed in RIPA buffer containing protease inhibitors (phenylmethyl sulfonyl fluoride, 200 mM; aprotinin, 1 mg/ml; trypsin/chymotrypsin inhibitor, 1 mg/ml; leupeptin, 1 mg/ml; pepstatin A, 1 mg/ml) (Sigma Chemical Company, St. Louis, MO). The lysate was sonicated for 10 sec (on ice) and stored at minus 20° C until Western blot analysis was performed. Prior to blotting, samples were thawed and 50 µl of lysate was added to 50 µl of 2% SDS and boiled for 5 min. Twenty microliters of each sample were then applied to a 12.5% PAGE electrophoresis gel (run for 35 min at 200V) and transferred to Immobilon™-P membrane (Millipore, Bedford, MA). The membrane was blocked with 5% nonfat dry milk for 2 hr. Subsequently, the membrane was washed with Tris-buffered saline-tween (TTBS) and incubated overnight with 1 µg/ml rabbit anti-IL-2 anti-serum (Santa Cruz Biotechnology, Santa Cruz, California) at 4 ° C. The membrane was again washed with TTBS and incubated with 0.16 µg/ml peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, California). Excess peroxidase was removed by washing the membrane with TTBS and with one final wash with tris-buffered saline (TBS). Finally, the membrane was soaked in Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, California) for 1 min and exposed to X-ray film.

Immunofluorescent antibody staining for membrane associated IL-2

One million RD995 cells transfected with IL-2tm were washed twice in PBS and incubated one ice for 30 min with rabbit anti-IL-2 antiserum (10 µg/ml) (Santa Cruz Biotechnology, Santa Cruz, California) or with monoclonal mouse anti-flag antibody (10 µg/ml) (Stratagene, La Jolla, CA). Cells were then washed with cold PBS+1% sodium

azide and incubated for 30 min with anti-rabbit Alexa 568 (10 μg/ml) (Molecular Probes, Eugene, OR) or with anti-mouse Alexa 488 (10 μg/ml), respectively. Parental RD995 cells and RD995 transfected with empty vector were similarly stained as negative controls. The cells were permeablized with 0.1% Triton X-100 (Bio-Rad, Richmond, CA) and 0.1% sodium citrate buffer incubated on ice for 2 minutes. After permeablizing the cell membrane, TO-PRO3™ (1 μM) (Molecular Probes, Eugene, OR) was added to stain the nucleus. Cells were then fixed in 1% paraformaldehyde for 30 min at room temperature. An Olympus Fluoview 200 laser scanning confocal microscope was used to visualize the subcellular localization of IL-2 in cells.

Evaluation of growth of RD995 cells transfected with IL-2tm in C3H/HEN mice

RD995 is a murine spindle cell skin cancer derived from an uv-irradiated C3H/HEN mouse. Groups of 10 C3H/HEN mice (Charles River Laboratories, Wilmington, MA) were implanted subcutaneously with 10^6, 5 x 10^5 or 10^5 IL-2tm transfected RD995. Equivalent numbers of pCMV2b transfected tumor cells or parental tumor cells were implanted to serve as controls. The maximum cross sectional dimension of each tumor were measured every other day with calipers and the area of the tumor was calculated.

PCR analysis of IL-2tm gene expression in excised RD995 tumors

On day 40 of tumor growth, mice were sacrificed and tumors excised to evaluate IL-2tm gene expression. Total RNA was extracted using TRI reagent and RT-PCR was

performed using IL-2tm primers that spanning the fusion gene product as previously described.

Tritiated thymidine ([3H]-Tdr) uptake by transfected RD995 tumors in vitro

In order to measure whether IL-2tm transfection altered tumor proliferation, 10^5 RD995 tumor cells (transfected either with IL-2tm, pCMV2b or 10^5 nontransfected RD995) were placed into each well of a 96-well tissue culture plate (24 replicates). Cells were incubated with 10 µl of [3H]-Tdr (50 µCi/ml) per well (NEN™ Life Science Products, Boston, MA) at 37° C for 48 hr. A PHD™ Cell harvester (Cambridge Technology Inc., Cambridge, MA) was used to harvest cellular DNA onto glass fiber filters (Gelman Sciences Inc., Ann Arbor, MI) and to wash away unincorporated [3H]-Tdr. The filter paper was allowed to air dry at room temperature for 4 hr, placed in 2 ml of scintillation fluid (Perkin Elmer Life Sciences, Boston, MA) and counted for one minute/sample on a 2500 TRI-CARB liquid scintillation analyzer (Perkin Elmer Life Sciences, Boston, MA).

Evaluation of biologic activity of IL-2tm using the IL-2 dependent cell line CTLL-20

In order to test whether the tumor cell expressed IL-2tm protein was biologically active, IL-2tm transfected tumor cells were lysed and added to the IL-2 dependent T cell line CTLL-20. Lysis of 5 X 10^6 RD995 cells transfected with IL-2tm or pCMV2b was accomplished by snap freezing in liquid nitrogen. The membrane portion of the lysate was separated from the cytosol by centrifugation for 15 min at 12,000xg. The cytosol

portion was placed into another tube and the membrane portion was reconstituted in an equivalent volume (0.5 ml) of RPMI media. One hundred microliters of membrane lysate or cytosol lysate (serial two-fold dilutions) were placed into a 96 well plate (in triplicate). One hundred thousand CTLL-20 cells were added to each well. CTLL-20 is an IL-2 dependent cell line derived from a C57BL/6 mouse (gift from D. Keith Bishop, University of Michigan Medical School). Prior to starting this experiment, IL-2tm expression in transfected RD995 cells was verified using RT-PCR and primers that span the IL-2 gene and transmembrane domain as previously described. A positive control consisted of serial dilutions of 200 IU recombinant hIL-2 (Chiron, Emeryville, CA). The plates were allowed to incubate at 37° C for 48 hr, and then each well was pulsed with 10 μl of [3H]-Tdr (50 μCi/ml) for 24 hr. After harvesting, samples were evaluated by scintillation counting.

RESULTS

Construction of IL-2tm

To test the hypothesis that membrane expression of IL-2 would lead to activation of T-cells infiltrating into cancers, it was necessary to create a fusion gene containing IL-2 and a transmembrane domain. The gene for hIL-2 (plasmid VR1103) was acquired as a gift from Vical Inc. hIL-2 was removed from pVR1103 because the flanking sequences of the hIL-2 gene in the VR1103 vector were unknown. Restriction enzymes PstI and BamHI were used to cut the hIL-2 gene from the plasmid. The gene was isolated on a 1% agarose gel (Figure 1). The expected hIL-2 size is 339 bp plus 217 extra base pairs from pVR1103 (total of 616 bp).

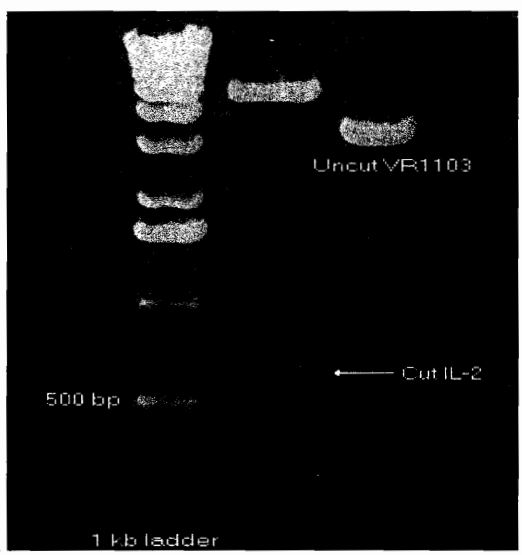

Figure 1. Plasmid VR1103 was cut with restriction enzymes PstI and BamHI. Lane 2 (see arrow) shows the faint hIL-2 band of 616 base pairs. hIL-2 DNA was then isolated from the 1% agarose gel using the GeneCAPSULE™ method.

In order to identify the stop codon in hIL-2, hIL-2 was ligated into cloning vector pBluescript KS to facilitate sequencing. pBluescript KS was cut with restriction enzymes PstI and BamHI. The gel isolated hIL-2 band (from Figure 1) and the gel isolated cut pBluescript KS were ligated together. DNA based primers for hIL-2 were used to perform PCR on the ligated pBluescript KS+hIL-2. Figure 2 illustrates successful ligation of hIL-2 gene into pBluescript.

pBluescript KS was sequenced (Figure 3) to identify the stop codon. The stop codon was removed by PCR directed mutagenesis. DNA based primers were designed to amplify the hIL-2 gene without the stop codon. Restriction sites were also incorporated onto the ends of the primers to maintain proper orientation and reading frame of the hIL-2 gene during ligation. Following DNA based PCR, the modified IL-2 gene was isolated from a 1% agarose gel (Figure 4).

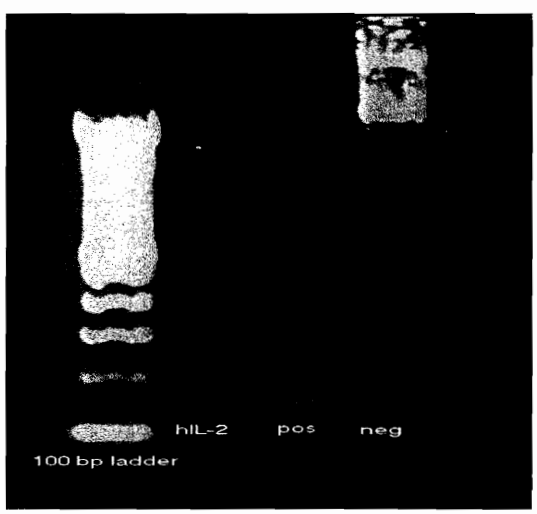

Figure 2. hIL-2 was ligated into cloning vector pBluescript KS. DH5α™Competent cells were transformed with hIL-2/pBluescript KS and screened for recombinant plasmids by lacZ color selection. hIL-2 DNA insertion was then verified by DNA based PCR using primers directed toward hIL-2. PCR was run for 32 cycles resulting in a 155 base pair product. Plasmid VR1103 was used as a positive control and uncut pBluescript KS was used as a negative control.

CACAAACAGT**GCACCT**ACTTCAAGTTCTACAAAGAAAACACAGCTACA
ACTGGAGCATTTACTGCTGGATTTACAGATGATTTTGAATGGAATTAATA
ATTACAAGAATCCCAAACTCACCAGGATGCTCACATTTAAGTTTTACATG
CCCAAGAAGGCCACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAAC
TCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGCAAAAACTTTCA
CTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAA
CTAAAGGGATCTGAAACAACATTCATGTGTGAATATGCTGATGAGACAG
CAACCATTGTAGAATTTCTGAACAGATGGATTACCTTTTGTCAAAGCATC
ATCTCAACACTGACTTGA**TAATTAA**GTGCTTCCCACTTAAAACATATCAG
GGATCTCGACTCTAGAGGATCAAC

Figure 3. T3 promoter based primers were used to sequence hIL-2. Bold underlined text indicates beginning and end of the hIL-2 gene. The TAATTAA stop codon was later removed by PCR directed mutagenesis.

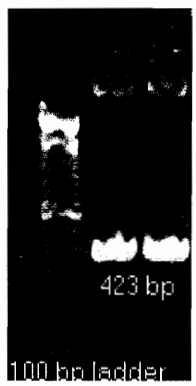

Figure 4. Primers were designed to incorporate restriction sites BamHI (5' GGA TCC 3') on the sense strand and EcoRI (5' GAA TTC 3') on the antisense strand in order to facilitate insertion of hIL-2 into plasmid HTAAA91 that contains the transmembrane domain Fcε-γ. Herculase™ Enhanced DNA Polymerase was used to amplify hIL-2 for 30 cycles. The 423 base pair band was isolated from the gel.

The hIL-2 gene and Fcε-γ transmembrane domain were each cut with BamHI and EcoRI. The hIL-2 (minus the stop codon) construct was ligated upstream and in frame with the transmembrane domain Fcε-γ derived from the plasmid HTAAA91. Fcε-γ is an accessory signaling protein of the Fcε receptor that has a minimal extracellular domain. DNA sequencing verified that hIL-2 (minus the stop codon) was upstream and in frame with Fcε-γ (Figure 5).

Lastly, in order to express hIL-2 on the surface of tumors, a mammalian expression vector was selected to deliver the hIL-2 +Fcε-γ fusion gene. pCMV2b contains a cytomegalovirus promoter along with neomycin and kanamycin resistance selection markers. Additionally, pCMV2b also encodes an N-terminal FLAG® tagging protein. We included the FLAG protein for use in identifying hIL-2 expression on the surface of tumors.

TCC**GCACCT**ACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGA
GCATTTACTGCTGGATTTACAGATGATTTTGAATGGAATTAATAATTAC
AAGAATCCCAAACTCACCAGGATGCTCACATTTAAGTTTTACATGCCC
AAGAAGGCCACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTC
AAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGCAAAAACTTTCAC
TTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAA
CTAAAGGGATCTGAAACAACATTCATGTGTGAATATGCTGATGAGACA
GCAACCATTGTAGAATTTCTGAACAGATGGATTACCTTTTGTCAAAGCA
TCTCAACACTGACTGAATTCGGCACGAGGGCCGATCTCCAGCC**AGAT**
GATTCACCAGCAGTGGTCTTGCTCTTACTCCTTTTGGTTGAACAAGCA
GCGGCCCTGGGAGAGCCTCA

Figure 5. T3 promoter based primers were used to sequence hIL-2 + Fcε-γ. Bold underlined text shows the beginning of hIL-2 and the beginning of the transmembrane domain Fcε-γ. Sequencing verifies that the stop codon from hIL-2 has been removed and that hIL-2 was upstream and in frame with the transmembrane domain.

Restriction enzymes ApaI and BamHI were used to cut HTAAA91 and pCMV2b. The IL-2tm (hIL-2+ Fcε-γ) construct was then ligated into pCMV2b as previously described. IL-2tm was sequenced to verify proper insertion into the expression vector. DNA sequencing (Figure 6) demonstrated that IL-2tm was ligated in the proper reading frame.

Screening for IL-2tm and pCMV2b mRNA and protein expression in RD995 tumor cells

Optimal conditions for transfection of RD995 tumor cells with IL-2tm contained within pCMV2b were established experimentally. We subsequently evaluated the ability of pCMV2b vector containing IL-2tm to induce mRNA and protein expression. Total RNA was extracted from transfected RD995 tumor cells (2 μg IL-2tm and 10 μl lipofectin). The RNA was treated with DNase to remove any residual plasmid derived DNA. After reverse transcription, DNA based primers, spanning the hIL-2+ Fcε-γ fusion gene were used to amplify the IL-2tm fusion gene by DNA based PCR. Figure 7 demonstrates that IL-2tm mRNA was easily detected in transfected RD995 cells. Control cells transfected with pCMV2b did not express this message.

Expression of IL-2tm protein in transfected RD995 cells was evaluated by Western blot analysis. Cell lysates were harvested on days 1-5 after transfection and analyzed for hIL-2 protein. Figure 8 shows that hIL-2 (18kD) and hIL-2 + transmembrane domain (27kD) are both present. Recombinant hIL-2 from Chiron was used as a positive control. The native IL-2 produces a band of 15 kD, but multimeric bands are also seen at 30 kD and 45 kD.

TGGAGCTCCCCGCGGTGGCGGCCGCCCC**ATGGATTA**CAAGGATGAC
GACGATAAGAGCCCGGGCGGATCCGCACCTACTTCAAGTTCTACAAAG
AAAACACAGCTACAACTGGAGCATTTACTGCTGGATTTACAGATGATT
TTGAATGGAATTAATAATTACAAGAATCCCAAACTCACCAGGATGCTC
ACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAACTGAAACATCTT
CAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTA
GCTCAAAGCAAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAAT
ATCAACGTAATAGTTCTGGAACTAAAGGGATCTGAAACAACATTCATG
TGTGAATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGA
TGGATTACCTTTTGTCAAAGCATCTCAACACTGACTGAATTCGGCACGA
GGGCCGATCTCCAGCCCAAG**ATGATT**CCAGCAGTGGTCTTGCTCTTA
CTCCTTTTGGTTGAACAAGCAGCGGCCCTGGGAGAGCCTCAGCTCTGCT
ATATCCTGGATGCCATCCTGTTTCTGTATGGAATTGTCCTCACCCTCCTC
TACTGTCGACTGAAGATCCAAGTGCGAAAGGCAGCTATAACCAGCTAT
GAGAAATCAGATGGTGTTTACACGGGCCTGAGCACCAGGAACCAGGA
GACTTACAAGACTCTGAAGCATGAGAAACCACCACAGTAGCTTTAGAA
TAGATGCGGNCATATTCTTCTTTGGCTTCTGGGTCTTNCAGCCCTCATG
GGTGGCATCACATATGCCTGCATGCCATTAACACCAGCTGGGCCTACC
CCTATNATGGATCCTGNGTCCTAAATTATATACACCCAGNGGGTCTGG
AGCTCCCCGCGGTGGCGGCCGCCCCATGGATTACAAGGATGACGACGA

Figure 6. Sequence analysis demonstrates that IL-2tm has been inserted in the correct reading frame into expression vector pCMV2b. The upper bold underlined text indicates the initiation start site and the second bold underlined sequence identifies the beginning of the transmembrane domain.

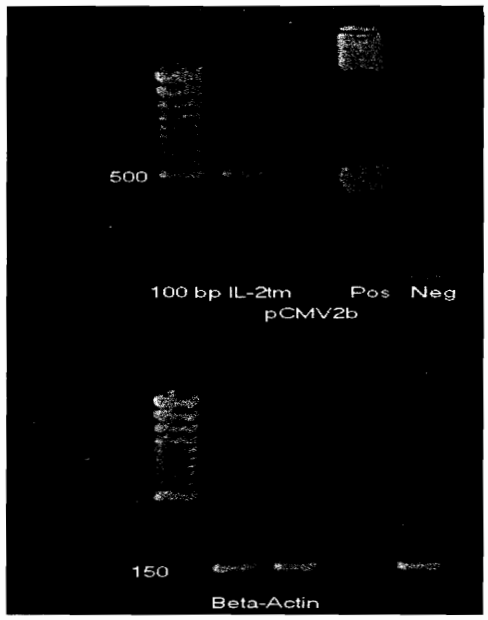

Figure 7. IL-2tm mRNA expression in RD995 cells transfected with IL-2tm or pCMV2b alone. Total RNA was extracted using TRI-REAGENT and treated with DNase to remove any residual plasmid DNA prior to reverse transcription. Primers were designed to span the hIL-2 gene and the transmembrane domain with an expected 500 base pair PCR product. This gel demonstrates that mRNA expression is present in cells transfected with IL-2tm, but not empty vector (pCMV2b).

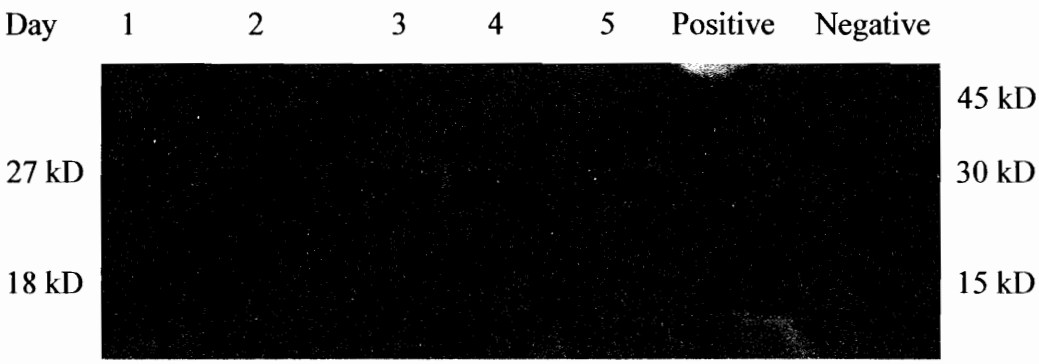

Figure 8. Western Blot analysis of IL-2tm expression. 10^6 RD995 cells were transfected with IL2-tm. 10^6 cells were harvested days 1-5 after transfection and washed in PBS and lysed in RIPA buffer. The lysates were applied to a 12.5% PAGE electrophoresis gel. After electrophoretic transfer to a membrane, and blocking steps, membranes were stained with 1 µg/ml polyclonal rabbit anti-IL-2 antibody. Bands were visualized using Luminol on x-ray film. Recombinant hIL-2 from Chiron was used as a positive control. The positive control shows three expected bands of IL-2 (45 kD, 30 kD and 15 kD) on the right side of blot. Non-transfected RD995 cells were used as a negative control. IL-2tm appears to generate both hIL-2 bound to transmembrane (27kD) and free hIL-2 (18kD) on left side of blot.

Immunofluorescent antibody staining for surface bound hIL-2 in RD995 tumor cells

Laser confocal microscopy was utilized to demonstrate that IL-2 was being expressed on the surface of transfected RD995 cells. RD995 cells transfected with IL-2tm or pCMV2b (10^6 cells) were stained with anti-hIL-2 or anti-FLAG (Figures 9 and 10). TO-PRO3™ nuclear stain was applied to the cells after they were stained with anti-hIL-2 or anti-FLAG. Untransfected RD995 or RD995 stained with secondary antibody alone were used as negative controls and also showed no staining (data not shown).

Evaluation of growth of RD995 cells transfected with IL-2tm in C3H/HEN mice

We hypothesized that expression of IL-2tm protein on tumor cell surface membranes would alter immunologic recognition in mice. We therefore evaluated the growth of IL-2tm transfected RD995 tumor cells in syngenic mice. RD995 cells transfected with IL-2tm were implanted subcutaneously into groups of 10 C3H/HEN mice (10^6, 5 x 10^5 or 10^5 tumor cells per mouse). Mice implanted with equivalent numbers of nontransfected RD995 cells or empty vector (pCMV2b) transfected RD995 cells were used as controls. Prior to implantation of tumors, IL-2tm mRNA expression in transfected RD995 cells was verified using RT-PCR and primers that span the IL-2 gene and the transmembrane domain (Figure 11).

The maximum cross sectional area of each tumor was measured with calipers every other day. The results are shown in Figure 12. Growth of IL-2tm transfected tumors was markedly reduced compared to parental or empty vector transfected RD995.

A B

Figure 9. Laser confocal microscopy of IL-2tm and pCMV2b transfected RD995. Cells were stained with anti-hIL-2 antiserum and TO-PRO3™ (1μM/ml) in order to visualize the nucleus. (A) Red staining of cells transfected with IL-2tm demonstrated surface membrane expression of IL-2. (B) RD995 transfected with empty pCMV2b vector indicates no hIL-2 expression.

A B

Figure 10. Laser confocal microscopy of FLAG-stained RD995 transfected with IL-2tm or pCMV2b. (A) Green staining demonstrates FLAG antigen expression in RD995 transfected with IL-2tm. (B) RD995 transfected with pCMV2b demonstrated no staining.

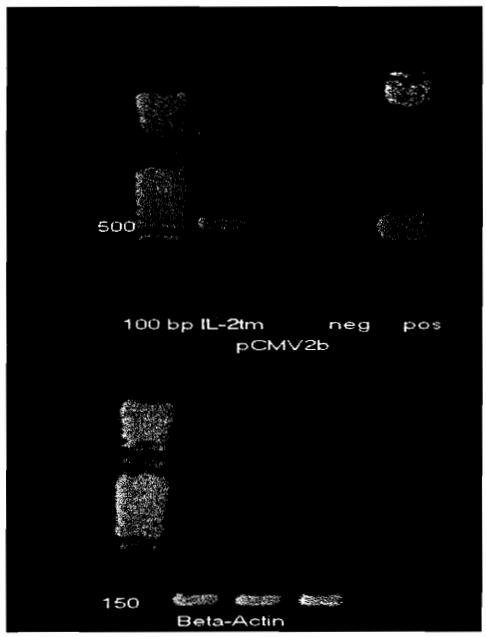

Figure 11. Prior to subcutaneous implantation of RD995 transfected with IL-2tm or pCMV2b, tumor cells were assayed for mRNA expression of IL-2tm. Primers designed to span the hIL-2 gene and the transmembrane domain generated the expected 500 base pair product. This gel demonstrates that mRNA expression is present, which was absent from parental tumor (neg) and empty vector transfected RD995 (pCMV2b).

The experiment was terminated on day 40 due to excessive size of tumors in control mice. On day 40, tumors in mice implanted with 10^6 IL-2tm RD995 cells showed reduced growth (52% smaller than in mice bearing pCMV2b RD995 and 59% smaller than parental RD995 tumor cells) (Figure 12). The group implanted with 5 x 10^5 IL-2tm RD995 tumor cells also showed reduced tumor growth (31% smaller tumors than the pCMV2b RD995 group and 30% smaller tumors than the parental RD995 group). No tumors grew in any groups of mice implanted with only 10^5 cells (data not shown). On day 40, mice from each group were sacrificed, the tumors excised and assayed for persistence of IL-2tm transgene expression. No residual IL-2tm mRNA could be detected in any tumors harvested on day 40, suggesting IL-2tm was lost prior to this time (data not shown).

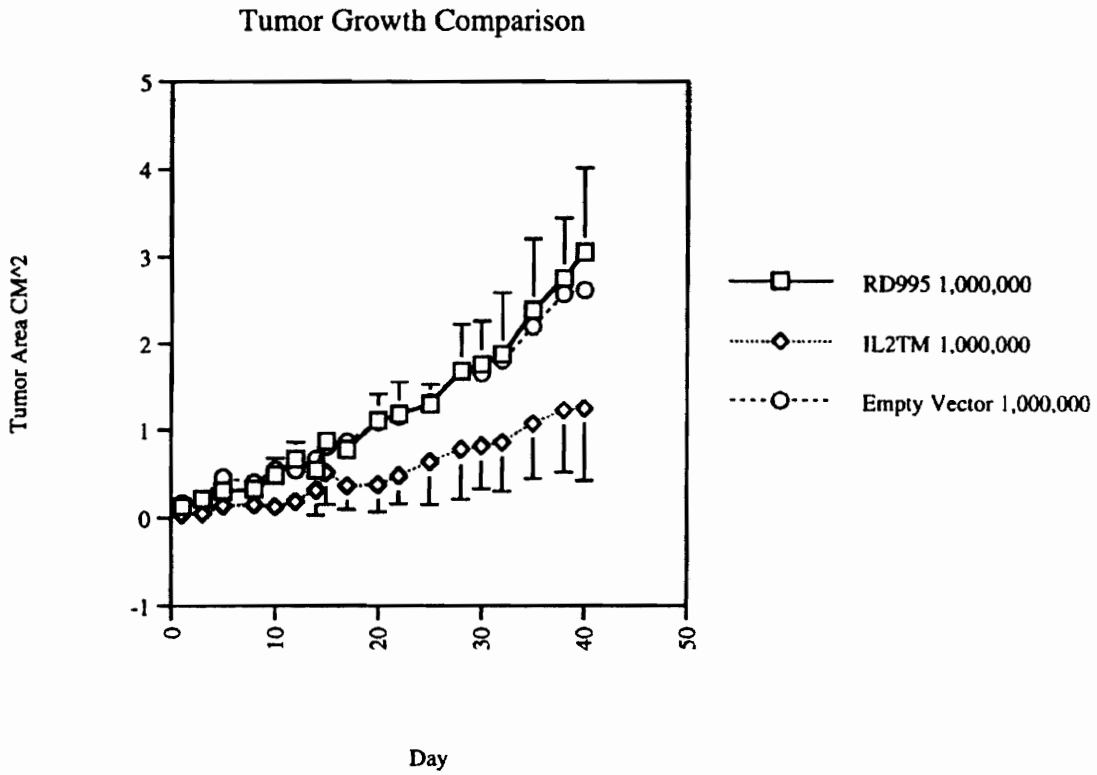

Figure 12. Growth of subcutaneous tumors in C3H/HEN mice injected with 10^6 RD995 transfected with IL-2tm, RD995 transfected with pCMV2b (empty vector) or RD995. The difference in growth rates between IL-2tm and the control groups was significant (p=0.011) by ANOVA.

Evaluation of the effect of IL-2tm and

pCMV2b or RD995 proliferation

The previous experiment demonstrated that RD995 cells transfected with IL-2tm grow at a slower rate than RD995 transfected with pCMV2b or parental RD995. A trivial explanation for this could be that tumor cells transfected with IL-2tm might have decreased proliferative potential due to some nonspecific cellular effect of the transgene. An in vitro thymidine incorporation assay was used to evaluate this possibility. This experiment showed that RD995 transfected with IL-2tm or pCMV2b had similar

thymidine incorporation as nontransfected RD995 (mean plus or minus SD of 24 wells) (Figure 13).

Evaluation of biological activity of IL-2tm

expressed in transfected RD995

The biological activity of IL-2tm was analyzed utilizing a murine cytotoxic T cell line that is dependent on IL-2 for growth. Recombinant human IL-2 is known to result in increased proliferation of CTLL-20 (measured by incorporated [3H]-TdR).[20-21]

A standard curve was generated using human recombinant hIL-2. It was found that 17 IU of IL-2 stimulated a measurable proliferation of CTLL-20. The relationship between thymidine incorporation and hIL-2 concentration was almost linear between 17 and 200 IU. This assay showed that RD995 cells transfected with IL-2tm or pCMV2b failed to induce detectable proliferation of CTLL-20. This implies less than 17 IU of biologically active IL-2 was expressed per 10^5 tumor cells.

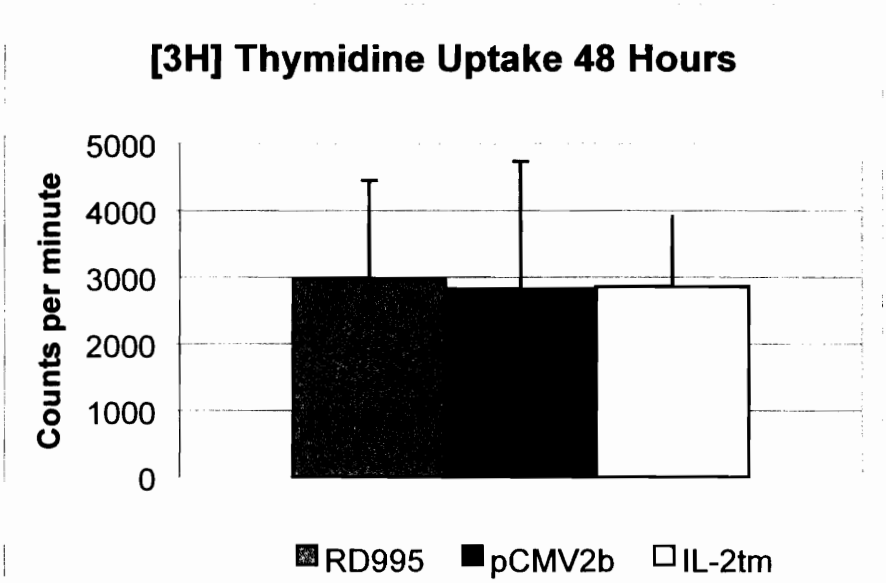

Figure 13. Analysis of thymidine incorporation in 10^5 RD995, RD995 transfected with pCMV2b or IL-2tm after 48hr in culture.

DISCUSSION

Using molecular genetic approaches, a fusion gene containing human interleukin 2 (hIL-2) and a transmembrane domain (IL-2tm) derived from Fcε-γ was created. The purpose of this construct was to provide a transmembrane anchor allowing hIL-2 to be expressed at the cell membrane of transfected cells, in close proximity to tumor infiltrating lymphocytes (TIL cells) and tumor specific antigens.[11] The transgene was subsequently cloned into a mammalian expression vector (pCMV2b). The correct orientation of the transgene was verified by DNA sequencing.

IL-2tm was transfected into murine RD995 tumor cells. Cells were evaluated for IL-2tm mRNA and protein expression. RT-PCR was performed with DNA based primers that span the IL-2 gene and the Fcε-γ gene and were used to distinguish the transgene from any native IL-2 mRNA (if present). This sequence has no known mammalian homologues. DNase treatment was used to exclude carry over of the IL-2tm containing pCMV2b plasmid. Using this RT-PCR assay, we found that IL-2tm mRNA was expressed in transfected RD995 tumor cells but not in pCMV2b transfected or parental RD995 cells. Western blot analysis was performed to detect IL-2tm protein in transfected RD995 cells. IL-2tm was detectable in RD995 transfected with IL-2tm (a 27kD protein). Protein expression persisted for at least 5 days following transfection. Interestingly, two bands of IL-2 were seen on the Western blot. One band correlated with the expected 27 kD IL-2tm protein. The other band correlated with IL-2 (18kD), which

suggests cleavage of the IL-2tm from the transmembrane anchor. The parental RD995 tumor was shown not to produce any IL-2 protein. Further investigation will be necessary to determine the exact site of cleavage and the enzyme responsible. IL-2tm protein expression on the surface of cells was confirmed by immunofluorescent staining of IL-2tm transfected RD995 cells. Anti-hIL-2 antiserum staining of RD995 cells transfected with IL-2tm showed that hIL-2 was expressed on the surface membrane by laser confocal microscopy. Anti-FLAG antibody was used to visualize the FLAG neoprotein that had been added to the N-terminus of IL-2tm. Immunofluorescent staining for FLAG also confirmed expression at the surface of IL-2tm transfected RD995. Parental RD995 or RD995 transfected with pCMV2b (empty expression vector) did not exhibit surface expression of FLAG or IL-2 protein.

To test the biologic activity of the transgene, groups of 10 syngenic (C3H/HEN) mice were injected with 10^5, 5 x 10^5 or 10^6 tumor cells subcutaneously. Mice implanted with equivalent numbers of pCMV2b or parental tumors served as controls. Expression of the transgene in IL-2tm transfected RD995 cells was verified at the start of the experiment. RD995 tumor cells (10^6 or 10^5 /mouse) transfected with IL-2tm grew more slowly than RD995 transfected with pCMV2b or nontransfected control RD995 implanted tumors after implantation into syngenic animals. The experiment was terminated on day 40 due to the large size of tumors in control mice. To assess whether the transgene was still expressed, mice were sacrificed and excised tumors were assayed for mRNA expression of IL-2tm or pCMV2b. It was found that mRNA expression had been lost. A trivial explanation for the decreased proliferation rate of RD995 cells transfected with IL-2tm (in C3H/HEN mice) might be due to nonspecific effects of the

transgene. Tritiated thymidine incorporation into transfected and nontransfected tumor cells established that these cells proliferate at essentially at the same rate, thus excluding this possibility.

Biological activity of IL-2tm was analyzed by utilizing an IL-2 dependent cell line.[20-21] This assay (with a sensitivity of 17 U IL-2/well) was unable to detect bioactive IL-2 in 10^5 tumor cells. This finding most likely may indicate very low levels of IL-2tm expression. Alternately we have considered the possibility of expression of inactive IL-2tm protein. If further experiments determine that IL-2tm is indeed inactive, we would propose that conformational issues might play a role. Hydrophobicity plots (data not shown) suggest that the residual intracellular domain of Fcε-γ may be folded and externally expressed on the cell surface along with the IL-2 protein. This could sterically hinder IL-2 from interacting with the IL-2 receptor. Alternately, the cell lysis procedure may inactivate IL-2. These possibilities are currently being tested.

We have therefore commenced additional modifications of the IL-2 vector to optimize biologic function. First, IL-2tm was designed to include the FLAG tagging protein to facilitate identification and tracking of the IL-2tm protein. The FLAG sequence may alter the tertiary protein structure of IL-2tm. It is strongly immunogenic and could initiate immunological recognition of the IL-2tm protein in future multi-injection experiments. The FLAG sequence has therefore been removed. Second, IL-2tm contained extraneous amino acids that were part of the multiple cloning site in the expression vector, which have also been deleted. Third, the mammalian expression vector, pCMV2b, does not contain an enhancer.[15, 22] mRNA expression levels may be

greatly increased by using an expression vector that includes an enhancer in addition to a promoter. The effects of these design changes are currently being tested.

The mechanism that resulted in decreased growth of RD995 cells transfected with IL-2tm in C3H/HEN mice is also being further investigated. Additional tumor cell lines will be transfected with modified IL-2tm plasmid (modifications described in the previous paragraph) to evaluate growth potential in vivo. Histological studies of tumor infiltrating lymphocytes and studies of tumor-specific T cell activation are planned to help evaluate the mechanism of the antitumor effect.

The eventual goal of this project is to produce a gene construct that can be used in gene therapy of cancer in humans. Activated TIL cells may be able to destroy tumors by recognizing tumor-associated antigens. Furthermore, activated TIL cells may have the ability to migrate throughout a patient's body and destroy tumor metastases. By further developing the IL-2tm vector, we may be a step closer to testing a practical gene therapy reagent for use against human cancer.

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